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THE USE OF HUMAN SKIN FOR THE DETECTION OF ANTI-EPITHELIAL AUTOANTIBODIES

A DIAGNOSTIC AND PROGNOSTIC TEST*

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ABSTRACT

Human skin as test substrate in the indirect immunofluorescent test has been found to be superior to rabbit esophagus in that it is considerably more sensitive for the detection of antiepithelial antibodies. By using horizontal skin sections rather than conventional vertical sections, the accuracy and reliability of the test has been greatly enhanced. The test technique which we describe is simple to perform, and yields reproducible results.

There was a good correlation between the initial serum antiepithelial antibody titer and the activity of disease in patients with pemphigus and bullous pemphigoid. In following the course of the disease in five patients the activity of the skin affection was usually reflected by the serum antibody levels.

In 1964, Beutner and Jordon, using the indirect immunofluorescent technique, demonstrated that sera of patients with pemphigus vulgaris contain antibodies which in vitro bind to heterologous and homologous stratified squamous epithelia (1). This observation has been confirmed many times (2–5). Jordon *et al.* later demonstrated in sera of patients with bullous pemphigoid (pemphigoid) antibodies which bind to the basement membrane area of heterologous, homologous, and autologous stratified epithelia (6). The antibodies present in sera from patients with pemphigus or bullous pemphigoid are specific for the respective diseases and yield typical immunofluorescent patterns in the in vitro tests.

Animal stratified squamous epithelia rather than human skin has been the preferred substrate for detecting antiepithelial antibodies with the indirect immunofluorescent test. Some of the problems of using human skin as the substrate are: nonspecific fluorescence of the stratum corneum and dermis, quenching of immunofluorescence by skin with a high melanin content, and the need to use acanthotic epidermis (2). In this paper we describe a simple indirect immunofluorescent test technique which utilizes human skin as the substrate and obviates the problems noted above by cutting the skin sections horizontally. In the technique described we also use commercially available fluorescein tagged antihuman immunoglobulin.

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MATERIALS AND METHODS

Clinical diagnosis. The diagnosis of pemphigus was established by clinical examination and either by histologic examination of biopsy material or by the presence of acantholytic cells in Tzanck preparations. The diagnosis of pemphigoid was made by clinical examination, histologic examination of biopsy material, and by the patient's failure to respond to dapsone or sulfapyridine treatment. A drug reaction was excluded through history. A bullous skin eruption associated with malignancies was excluded by physical examination, laboratory tests and x-ray examinations. Diagnosis of dermatitis herpetiformis (D.H.) was made by clinical examination, compatible histology, and responsiveness to dapsone or sulfapyridine (7).

Preparations of tissue sections. Six millimeter punch biopsies were obtained either from normal skin of human volunteers or from six to 24 hour old human cadavers. For comparative studies the mucosa of the upper halves of rabbit esophagi was also used. The skin and the rabbit esophagi were frozen and stored in liquid nitrogen for up to

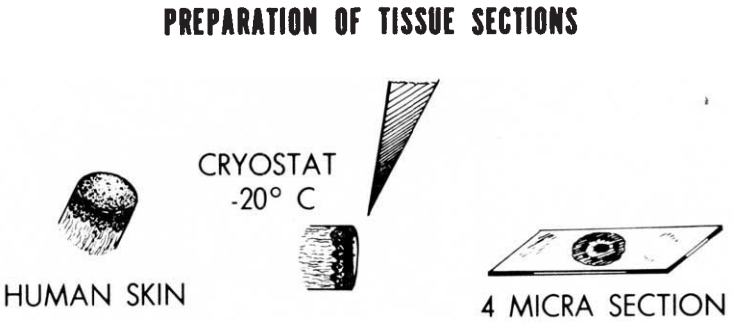

FIG. 1

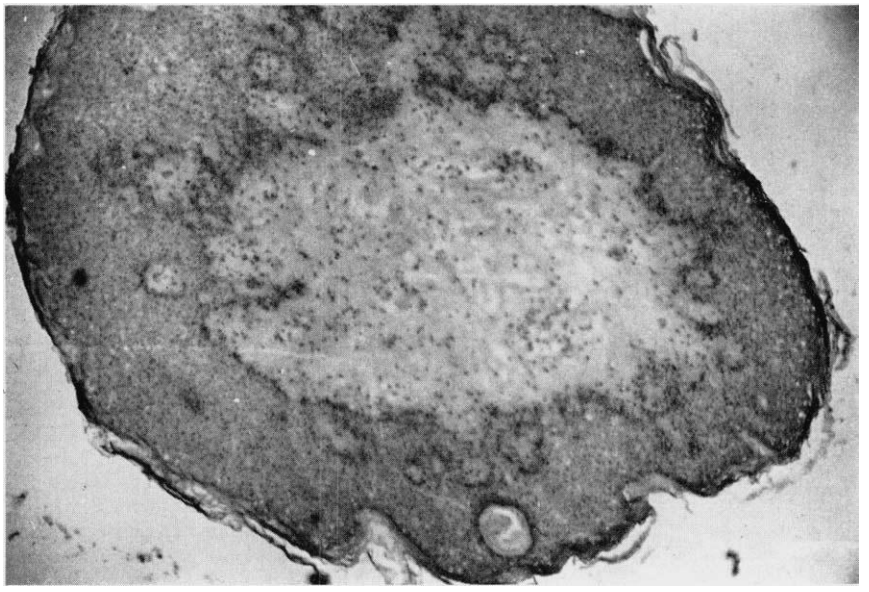

FIG. 2. Hematoxylin and eosin stain of human skin cut horizontally (parallel to skin surface).

one week. For sectioning in a cryostat they were embedded in OCT* media. Four micron thick horizontal (parallel to skin surface) sections were made from the skin and equal but transverse sections were made from the esophagi (Figs. 1 and 2). From each tissue sample about 100 sections were made. The sections were placed on glass slides and were either used on the same day or stored at −20° C. for use at a later time. Before use, the slides with the tissue sections were thawed and kept at room temperature for ½ to 1½ hours.

Immunofluorescent technique. Sera from patients with pemphigus, pemphigoid, and other skin diseases were kept frozen at −20° C. For testing, the thawed sera were diluted serially with isotonic saline starting with a 1:10 dilution. Undiluted sera were not used because they were often found to yield an unspecific epidermal fluorescence. The antibody titer is defined as the highest dilution of a serum which still gives a positive immunofluorescent test. For control purposes positive and negative control sera were always run with the unknown samples.

The indirect immunofluorescent staining technique of Weller and Coons (8) was used. The tissue section on the glass slide was covered with one to two drops of the serum dilutions and incubated at room temperature in a moist chamber for 30 mins. The serum was removed from the tissue section by immersion into three to four changes of a 0.15 M sodium phosphate buffer (pH 7.3) for 45 mins. The rinsed section was covered with one to two drops of fluorescein isothiocyanate tagged goat antiserum to human immunoglobulin (Ig)G, M or A.† (After reconstitution of the lyophilized antisera they were used in a 1:10 dilution made with phosphate buffer.) The tissue section was incubated at room temperature in a

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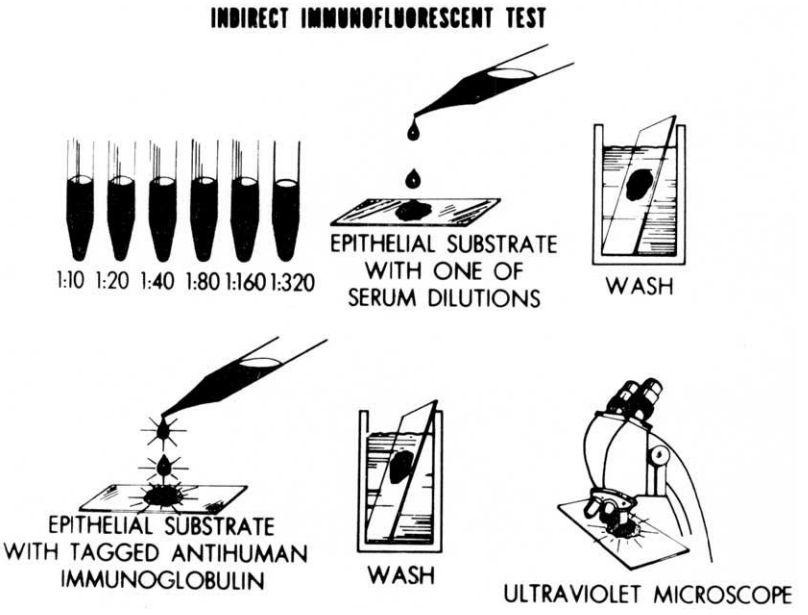

FIG. 3

moist chamber for 30 mins. The section was then washed again for 45 minutes with phosphate buffer and was covered with a drop of phosphate buffered glycerol (1:1 pH 7.3) and a cover slip applied. The slides were studied for immunofluorescence under ultraviolet light using a Leitz Orthoplan microscope (Fig. 3). The filters consisted of a BG-38 heat filter, a BG-12 exciter filter and a K-530 barrier filter.

Affinity of human skin and rabbit esophageal mucosa for antiepithelial antibodies. In order to assess the antibody affinity of human skin in comparison to rabbit esophageal mucosa many sera were tested several times using the same and different epithelia.

The stability of the epithelial antigens was determined on fresh sections of skin (including cadaver skin up to 24 hours old) and rabbit esophagus after storage of the sections at −20° C. for various periods at time.

The sensitivity of human skin and rabbit esophageal mucosa was determined by using four sera with known low autoantibody titers.

Reproducibility of the test. To determine the reproducibility of the test, immunofluorescent tests were performed with several sera using skin from different people and esophagi from different rabbits.

Two and occasionally three investigators interpreted the immunofluorescent pattern.

Stability of the stained slides. The presence or the absence of specific immunofluorescence was assessed immediately after performing the test. The slides were then kept at 4°–8° C. and checked for loss or fading of immunofluorescence at various time intervals.

Properties of the commercially prepared fluorescein tagged antisera. The molar fluorescein to protein ratios in the commercially obtained antihuman immunoglobulin preparations were determined with the method of Wells, Miller, and Nadel (9). The specificity of the antisera for gamma M, G, and A class of human antibodies was assayed by immunoelectrophoresis. The specificity of the immunofluorescent reaction was assessed by blocking experiments. The tissue sections, after having bound antiepithelial antibodies, were first incubated with unlabeled goat anti-human IgG and subsequently with the labeled goat anti-human IgG.

Correlation between disease activity and antibody titer. Initial autoantibody titers were determined with the sera of 25 patients with pemphigus and pemphigoid. The titers were correlated with the clinical activity of the disease. Also, five patients were observed during treatment and sequential titers were determined. When comparing sera titers in a given patient during the course of the disease all serum samples were tested simultaneously on the same epithelial tissue.

RESULTS

1. *Accuracy of the immunofluorescent test*

The sera of 165 patients with bullous and non-bullous diseases were tested for the presence or absence of antiepithelial antibodies. Normal human skin was used as the substrate. Figure 4 demonstrates the immunofluorescent staining pattern obtained with sera from pa-

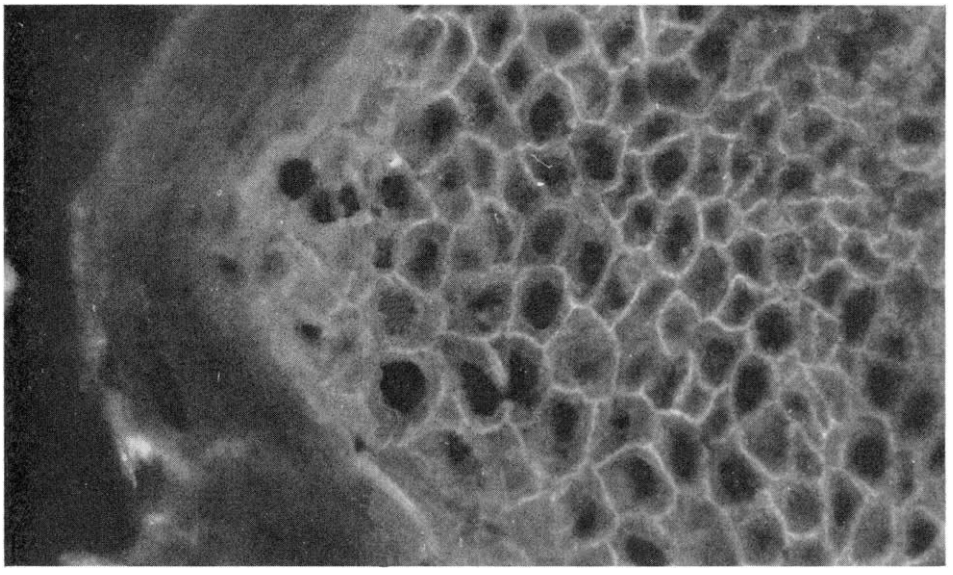

FIG. 4. Positive indirect immunofluorescent staining of epidermal cell boundaries using a 1:20 dilution of a serum from a patient with pemphigus. Stratum corneum at left.

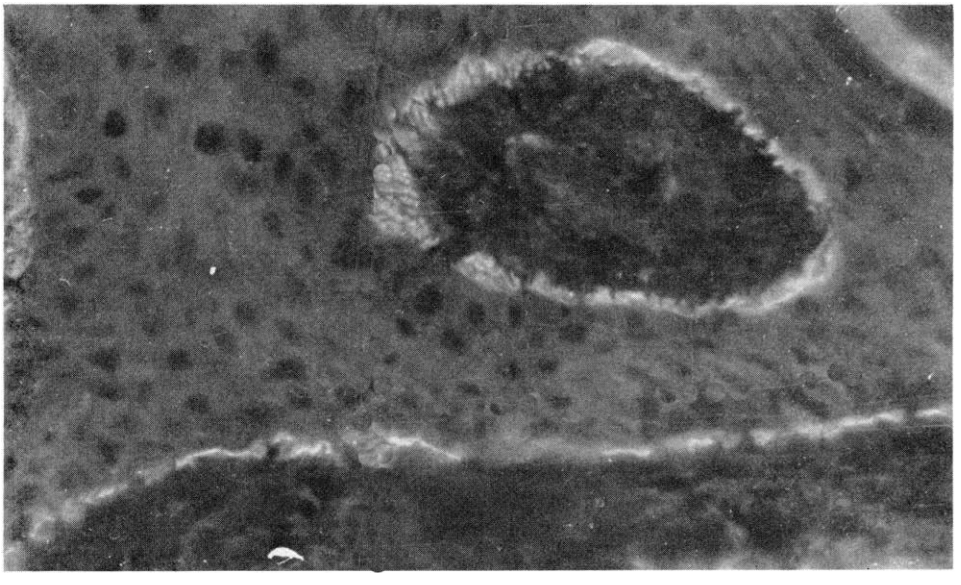

FIG. 5. Positive indirect immunofluorescent staining of basement membrane area of human skin using a 1:40 dilution of a serum from a patient with bullous pemphigoid. Dermis at bottom. Circle is immunofluorescence of basement membrane surrounding a dermal papilla (cut horizontally).

tients with pemphigus. The immunofluorescence outlines the boundaries of the epidermal cells ("pemphigus type"). Figure 5 shows the fluorescent staining of the basement membrane area which was obtained with the serum of a patient with bullous pemphigoid ("pemphigoid type"). In Figure 6 the nuclei of the epithelial cells fluoresce. The serum which induced this reaction was from a patient with lupus erythematosus.

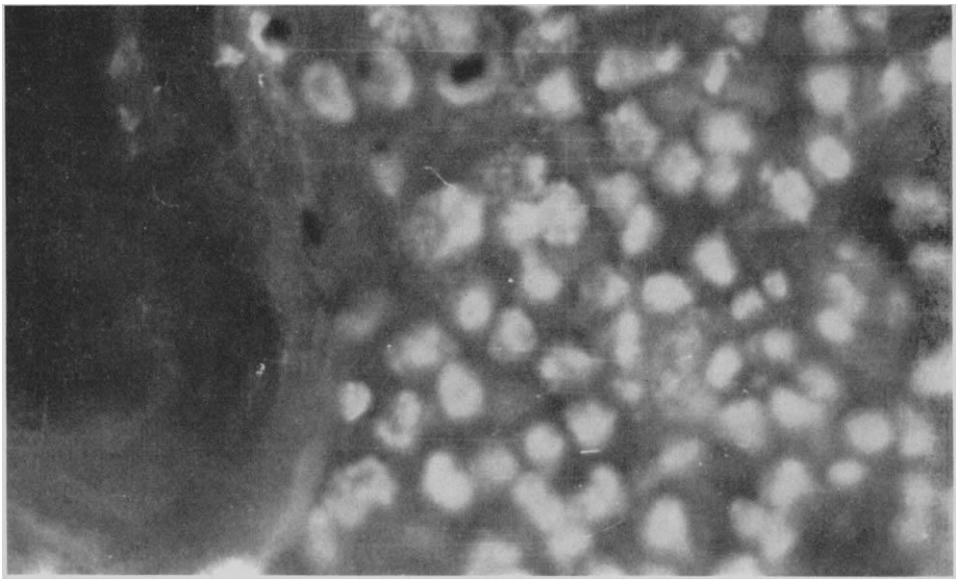

FIG. 6. Positive indirect immunofluorescent staining of epidermal cell nuclei using a 1:20 dilution of a serum from a patient with systemic lupus erythematosus. Stratum corneum at left.

The results of our tests with the sera from 165 patients are summarized in Table I. Two sera of 114 patients with nonvesiculobullous diseases contained antinuclear antibodies. None of the following sera contained antiepithelial antibodies: 12 from patients with dyshidrosiform or viral eruptions, six from patients with bullous erythema multiforme, five from patients with dermatitis herpetiformis, two from patients with Hailey-Hailey disease, and one from a patient with Darier's disease. Of 17 patients with pemphigus, 14 had serum antibodies which were bound to the epidermal cell boundaries of normal human skin. None of the pemphigus patients had antibodies which reacted with the basement membrane area. Seven of eight sera from patients with bullous pemphigoid had basement membrane antibodies. All antiepithelial antibodies (pemphigus, pemphigoid) were of the gamma G class. One serum of a patient with lupus erythematosus contained antinuclear antibodies of the gamma A class.

2. *Affinity of human skin and rabbit esophageal mucosa for antiepithelial antibodies*

a) *Stability.* The reactivity with antiepithelial antibodies of the "pemphigus type" was maintained in skin sections which had been kept for five weeks at −20° C. but had disappeared in rabbit esophagus sections kept for two weeks at this temperature. The reactivity with antiepithelial antibodies of the "pemphigoid type" was preserved in skin and esophagus for five and two weeks respectively.

b) *Sensitivity.* Four sera (Table II) with low antiepithelial antibody titers yielded negative immunofluorescent tests when rabbit esophageal mucosa was used. These tests were positive when human skin was used. None of the four sera from patients with pemphigus and pemphigoid which yielded negative tests when human skin was used, yielded positive tests with rabbit esophageal mucosa.

3. *Reproducibility of the test*

a) *Variability of different specimens of human skin and rabbit esophagus.* Sera tested several times using the same as well as different human skin showed that the highest serum dilutions which yielded a positive test were generally of the same order but were not identical each time they were tested. They usually varied by not more than one dilution but occasionally they varied by two dilutions (Table III). For example, serum A (Table

TABLE I

Results of indirect immunofluorescent test using normal human skin as substrate

Diagnosis	No.	Basement membrane area	Stratified epithelial cell boundaries	Epithelial cell nuclei
Control (nonvesiculobullous diseases)	114	0	0	2
Pemphigus	17	0	14	0
Bullous pemphigoid	8	7	0	0
Bullous E. M.	6	0	0	0
Hailey-Hailey	2	0	0	0
Darier's	1	0	0	0
Dermatitis herpetiformis	5	0	0	0
Other vesiculobullous disease (including dyshidrosiform and viral eruption)	12	0	0	0

TABLE II

Comparison of sensitivity of fresh human skin and rabbit esophagus for the detection of antiepithelial antibodies

Sera	"Pemphigus antibodies"		"Pemphigoid antibodies"	
	Skin	Esophagus	Skin	Esophagus
M	20*	Neg		
N	10	Neg		
O			20	Neg
P			20	Neg

* Highest dilution which yielded a positive test.

TABLE III

Antiepithelial antibody titers obtained with skin from different sources

Sera	Skin from different sources					Two skin specimens from same donor	
	1	2	3	4	5	6	6A
A	320*	320	160	160	80	160	320
B	160	160			160	160	320
C	160	160	320	160		320	640
D	320				80	160	

* Highest dilution which yielded a positive test.

III) yielded a positive immunofluorescent test at a 320 fold dilution and serum C yielded a positive immunofluorescent test at a 160 fold dilution when skin specimen 1 was used. However, when skin specimen 6 was used serum A yielded a positive immunofluorescent test at a 160 fold dilution and serum C a 320 fold dilution. There was no significant difference in the results when Negro skin was used.

Determinations made with several sera and using the same as well as different rabbit esophagi showed that the highest serum dilution yielding a positive test usually varied by not more than one dilution and only occasionally by two dilutions (Table IV).

b) *Determination of the serum antibody titer by different investigators.* When several observers evaluated the tests they differed frequently about one dilution variation in their interpretation of a positive or a negative test and only very rarely did observers differ in interpretation by two dilutions.

4. *Stability of the stained slides*

When titers were determined immediately after fluorescein staining and then left in the refrigerator the immunofluorescence did not fade for as long as one week.

5. *Properties of commercially labeled antisera*

The molar fluorescein to protein ratios of the various antisera were between 3.5 and 4.5 which indicates an optimal binding of fluorescein isothiocyanate to the protein. Immunoelectrophoretic analyses yielded a single line of precipitation when the antisera were tested with normal human sera. Previous incubation

TABLE IV

Antiepithelial antibody titers obtained with esophagi from different sources

Sera	Specimens from same rabbit		Specimens from different rabbits	
	1	2	3	4
A*	40†	40	160	80
B*	20	40	80	80
C*	80		80	160

* Same as A, B, and C in Table 3.

† Highest dilution which yielded a positive test.

of the tissue sections with unlabeled goat anti-human IgG prevented detectable binding of fluorescent anti-human IgG.

6. *Correlation between disease activity and antibody titer*

a) *Initial antiepithelial antibody titers and disease activity.* All sera from patients with active pemphigus or pemphigoid yielded a positive immunofluorescent test. With one exception patients with widespread active disease (pemphigus and pemphigoid) uniformly demonstrated serum antiephithelial antibody titers of 1:160 or higher. Patients with moderate involvement had serum antibody titers of 1:20 and higher. Patients with localized disease (especially mouth lesions) had serum titers of 1:10 or 1:20. Patients under adequate therapy with corticosteroids or in remission had low antibody titers or negative immunofluorescent tests (Table V).

b) *Sequential antiepithelial antibody titers correlated with changes in clinical activity.* One patient with widespread bullous pemphigoid and an initial serum titer of "pemphigoid type" antibodies of 1:160 had a titer of 1:20 when his skin disease improved with therapy. Three months after therapy began his skin showed only two small vesicles and his serum antibody titer was 1:10. Another patient with widespread pemphigus foliaceus was treated to hematologic toxicity with azathioprine (Imuran) with only slight clinical improvement. Her serum "pemphigus antibody" titer decreased from 1:320 to 1:80 during this time. She was then treated with high doses of prednisone (160 mg/day) for four weeks. Again her skin disease improved slightly and her serum antibody titer decreased to 1:40.

TABLE V

*Disease activity and initial autoantibody titers of sera from patients with pemphigus and bullous pemphigoid**

	No.	Serum titer							
		640†	320	160	80	40	20	10	Neg
Widespread									
Pemphigus	6	2	2	2					
Pemphigoid	5		1	3		1			
Moderate									
Pemphigus	2				1	1			
Pemphigoid	2				1		1		
Localized									
Pemphigus	5‡						2	3	
Pemphigoid	0								
Inactive and/or adequately treated									
Pemphigus	4							1	3
Pemphigoid	1								1

* Normal human skin used as substrate in the indirect immunofluorescent test.

† Highest dilution which yielded a positive test.

‡ Three of these patients had only mouth lesions.

The clinical serologic correlation of three other patients are shown in Figures 7–9. While under treatment with prednisone patient T.R.'s serum "pemphigus antibody" titer decreased (Fig. 7). This was followed by the complete healing of her skin. Her prednisone requirement could be markedly reduced without causing an exacerbation of the disease. She later had mouth lesions which regressed without treatment. She had a negative immunofluorescent test at this time. In the patient W.G. (Fig. 8) the skin changes had regressed completely, even before her "pemphigus antibody" titer had reached the low level of 1:10. An exacerbation of the skin disease was preceeded by a rise in antibody titer. In the patient M.N. (Fig. 9) the lowering of the "pemphigus antibody" titer was paralleled by the healing of the skin disease. For several weeks this patient developed no skin or mucous membranes lesions although his serum antibody titer was 1:40. When he again developed a few skin lesions his serum antibody titer remained unchanged.

DISCUSSION

Although the antiepithelial autoantibodies in sera of patients with pemphigus and pemphigoid are not species specific, human skin is the natural substrate they can react with. In most studies heterologous epithelia were used for the detection of such antiepithelial antibodies.

We found human skin to be more sensitive than rabbit esophagus because it allowed for the detection of lower serum antibody titers. In addition, human skin retained reactivity with these antibodies for a longer time than the epithelium of rabbit esophagus. The human skin was much easier to interpret for positive or

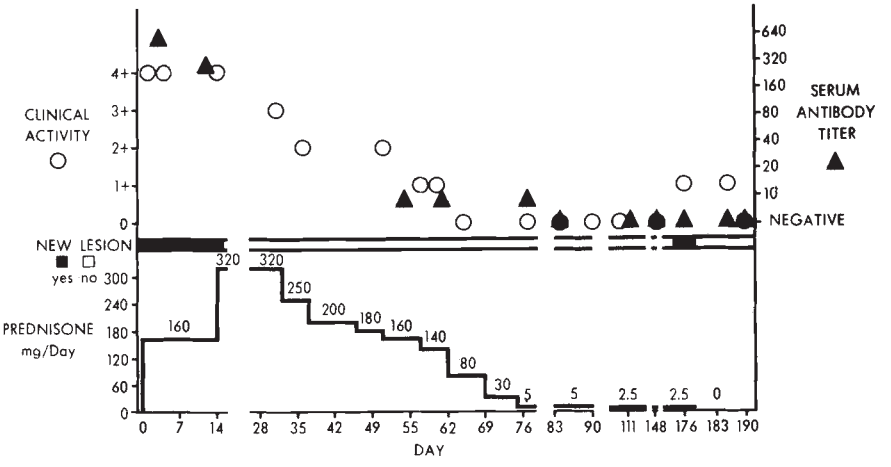

FIG. 7. Clinical-serologic-therapeutic correlation of patient (T.R) with pemphigus.

Clinical Activity

1+ One to four small lesions with or without mouth lesions or widespread lesions almost entirely healed.
2+ Few areas with several lesions or widespread lesions partly healed.
3+ Many large areas of involvement or widespread lesions with early signs of healing.
4+ Almost entire body covered with lesions.

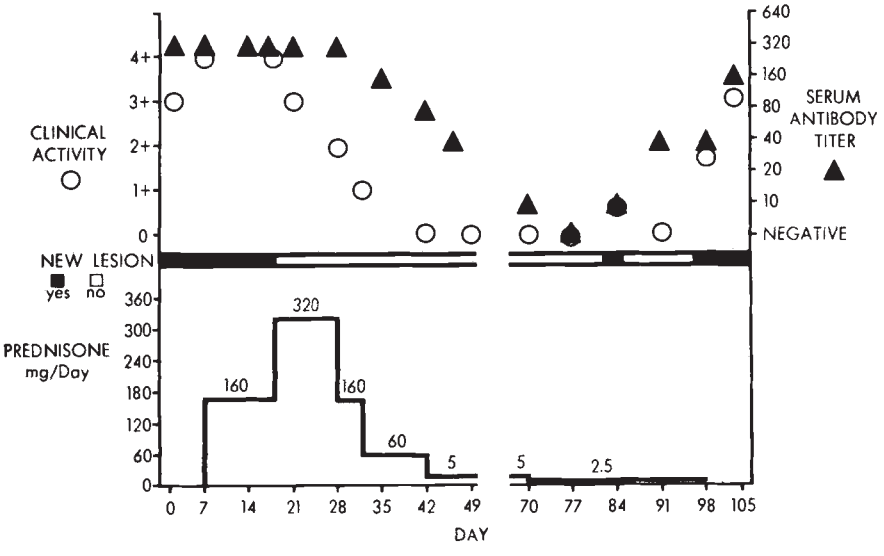

FIG. 8. Clinical-serologic-therapeutic correlation of patient (W.G.) with pemphigus.

negative antibody binding by means of the immunofluorescent test than was rabbit esophagus, especially when determining antibody titers. Human skin is also an attractive substrate to use since it is the most readily available material in most dermatologic facilities. However human volunteers are not essential since 24 hour old skin from cadavers or skin from amputated limb is as sensitive as fresh human skin.

In most of the previous immunofluorescent studies of bullous disease, skin was used as the substrate to detect in vivo bound antibody (direct immunofluorescent test) (10). Waldorf discussed some of the problems of using skin as the substrate for indirect immunofluorescent

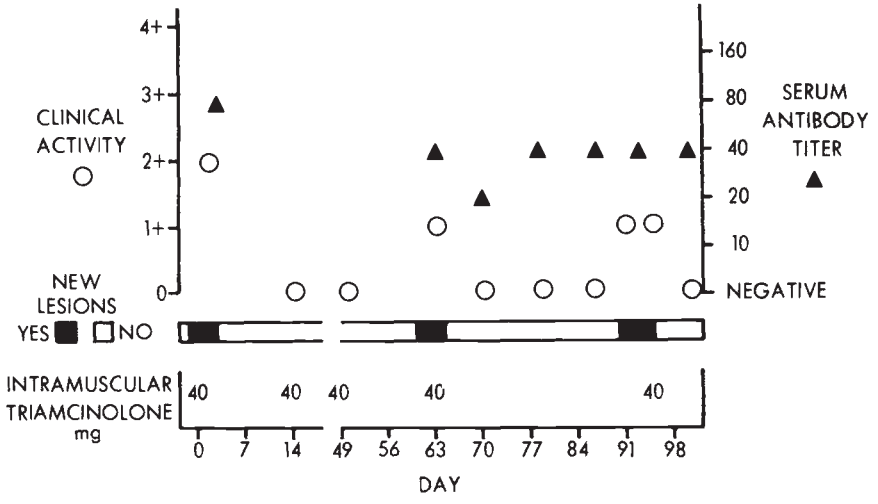

FIG. 9. Clinical-serologic-therapeutic correlation of patient (M.N.) with pemphigus.

staining; the nonspecific fluorescence of the stratum corneum and dermis, the quenching of immunofluorescence by skin with a high melanin content, and the need to use acanthotic epidermis (2). We have obviated these problems by cutting the skin sections horizontally. This gives an artifactually "acanthotic" epidermis and islands of superficial dermis surrounded by basement membrane within the epidermis. This thick epidermis and increased amounts of basement membrane also minimize the appearance of nonspecific fluorescence of the stratum corneum as well as the dermis. Although skin with high melanin content quenched fluorescence in the basal cell layer it had no effect on the fluorescence of the rest of the specimen. In accordance with the observations of others, we found that though "pemphigus type" immunofluorescence occurred throughout the entire epidermis, it was most prominent in the prickle cell layer.

We found that the commercially obtainable fluorescein isothiocyanate tagged goat anti-human immunoglobulin was as effective as our own preparations.

Our results show that neither of the two substrates, human skin or rabbit esophagus yielded identical results in regard to antibody titers. A one-fold difference in titer is common; two-fold differences are infrequent. To minimize variability, we suggest that comparisons of sera obtained from the same patient should all be tested together on one day and on the same substrate.

To our knowledge, the antiepithelial autoantibodies in all of the patients with pemphigus and pemphigoid are of the gamma G class.

None of the sera of our pemphigus patients had a concomitant antinuclear antibody as did several in Peck's study (5).

In accordance with Chorzelski *et al.* (4) and Peck *et al.* (5), we also observed a good correlation between the initial antiepithelial antibody titer and the activity of disease in patients with pemphigus. When following the clinical course of the diease and the autoantibody titers we found that the activity of the disease was usually reflected by the antibody titer levels. Therefore sequential titer determinations can be of predictive value for regression or for potential aggravation of the disease.

Peck *et al.* (5) found a poor correlation of serum antibody titers and the disease activity in patients with bullous pemphigoid. In contrast to this, we found the titer of basement membrane antibodies to correlate well with the activity of disease in our eight patients with pemphigoid. We treated one patient with bullous pemphigoid whose serum antibody titer decreased while his skin disease improved.

Several of our pemphigoid patients initially were diagnosed as suffering from dermatitis herpetiformis (D.H.). The histopathologic skin changes were compatible with either dermatitis herpetiformis, pemphigoid, or bullous erythema multiforme. Their skin disease did not improve with dapsone or sulfapyridine therapy but improved when they were treated

with corticosteroids. On this basis the diagnosis of pemphigoid was made. Subsequently the presence of "pemphigoid type" antiepithelial antibodies was detected in their sera. Published data indicate that to date over 100 patients with pemphigoid and D.H. have been studied for "pemphigoid type" serum antibodies. Sixty percent of patients with the clinical diagnosis of pemphigoid had such antibodies. However, their disease activity was not stated. In this report 100 percent of patients with active pemphigoid had antiepithelial antibodies. The higher incidence of such antibodies in our study may be explained by the use of the more sensitive human skin as assay substrate. Alternatively, the other studies may have included many patients with inactive disease. None of our patients who responded favorably to a treatment with dapsone or sulfapyridine had circulating "pemphigoid type" antibodies.

REFERENCES

1. Beutner, E. H. and Jordon, R. E.: Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescent staining. Proc. Soc. Exp. Biol. Med., *117:* 505, 1964.
2. Waldorf, D. S., Smith, C. W. and Strauss, A. J. L.: Immunofluorescent studies in pemphigus vulgaris. Arch. Derm., *93:* 28, 1966.
3. Beutner, E. H., Lever, W. F., Witebsky, E., Jordon, R. E. and Chertock, B.: Autoantibodies to pemphigus vulgaris. J.A.M.A., *192:* 682, 1965.
4. Chorzelski, T. P., von Weiss, J. and Lever, W. F.: Clinical significance of autoantibodies in pemphigus. Arch. Derm., *93:* 570, 1966.
5. Peck, S. M., Osserman, K. E., Weiner, L. B., Lefkovits, A. and Osserman, R. S.: Studies in bullous diseases. New Eng. J. Med., *279:* 951, 1968.
6. Jordon, R. E., Beutner, E. H., Witebsky, E., Blumenthal, G., Hale, W. L. and Lever, W. F.: Basement zone antibodies in bullous pemphigoid. J.A.M.A., *200:* 751, 1967.
7. Lever, W. F.: *Pemphigus and Pemphigoid.* Chas. C Thomas Co., Springfield, Ill., 1965.
8. Weller, T. H. and Coons, A. H.: Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Proc. Soc. Exp. Biol. Med., *86:* 789, 1954.
9. Wells, A. F., Miller, C. F. and Nadel, M. K.: Rapid fluorescein and protein assay method for fluorescent-antibody conjugates. Appl. Microbiol., *14:* 271, 1966.
10. Wright, E. T., Epps, R. L. and Newcomer, V. D.: Fluorescent antibody studies in pemphigus vulgaris. Arch. Derm., *93:* 562, 1966.